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(54) Title: TRANSGENIC ANIMAL PRODUCTION USING LOPU-DERIVED OOCYTES

(57) Abstract: Methods for generating transgenic non-human animals using oocytes derived from laparoscopic ovum pick up.

TRANSGENIC ANIMAL PRODUCTION USING LOPU-DERIVED OOCYTES

Background of the Invention

The field of the invention is methods for the development and propagation of transgenic animals.

Techniques to generate transgenic animals by the introduction of a recombinant DNA into zygotes, fetal cells, or oocytes are well known (reviewed by Wall, Theriogenology 45:57-68, 1996). Methods to develop transgenic animals carrying a gene fused to a tissue-specific promoter, such as milk, are also known (WO 93/25567). The use of transgenic animals carrying such transgenes makes it possible to produce desired polypeptides in the animals. These polypeptides can be produced in larger quantities and with less expense than those produced using more traditional methods of protein production in microorganisms or animal cells. Once transgenic animals are generated, their offspring can be used in efficient, tissue-specific production of desired polypeptides.

Summary of the Invention

The invention features methods for the generation of a transgenic animal using oocytes recovered through laparoscopic aspiration of follicles. This oocyte recovery procedure is also known as laparoscopic ovum pick up (LOPU), laparoscopic follicle aspiration, or laparoscopic oocyte aspiration. A transgene may be introduced into LOPU-derived oocytes, for example, by microinjection of a DNA construct or retroviral particles, or by retroviral infection (Wall, supra). The oocytes are then fertilized, and the formed zygote, or morulae or blastocyst grown from the zygote is transferred into a recipient.

In another method, a transgene is introduced into a zygote, or a cleaved embryo, morulae, or blastocyst formed from by culturing the zygotes by microinjection or infection, followed by transfer into a recipient. In yet another method, a transgenic animal may be generated by recovering oocytes from a transgenic founder, using the LOPU procedure, followed by fertilization and transfer to a recipient. Alternatively, non-transgenic oocytes may be recovered, using the LOPU procedure, and fertilized *in vitro* with transgenic semen to produce a transgenic animal.

Accordingly, in a first aspect, the invention features a method for generating a non-human transgenic animal, comprised of the following steps:

(a) recovering an oocyte from a donor animal by laparoscopic ovum pick up;

(b) *in vitro* maturing the oocyte;

(c) *in vitro* fertilizing the oocyte to produce a zygote;

(d) introducing a transgene into the zygote, or a cleaved embryo, morulae, or blastocyst formed by culturing the zygote;

(e) transferring the zygote, cleaved embryo, morulae, or blastocyst into a recipient animal; and

(f) allowing the zygote, cleaved embryo, morulae, or blastocyst to develop to term.

In a second aspect, the invention features a method for generating a non-human transgenic animal, involving:

(a) recovering an oocyte from a donor animal by laparoscopic ovum pick up;

(b) introducing a transgene into the oocyte to produce a transgenic oocyte;

(c) *in vitro* maturing the transgenic oocyte;

(d) *in vitro* fertilizing the transgenic oocyte to produce a zygote;

(e) transferring the zygote, or a cleaved embryo, morulae, or blastocyst formed from culturing the zygote into a recipient animal; and

(f) allowing the zygote, cleaved embryo, morulae, or blastocyst to develop to term.

In a third aspect, the invention features a method for generating a non-human transgenic animal, involving:

(a) recovering an oocyte from a donor animal by laparoscopic ovum pick up;

(b) *in vitro* maturing the oocyte;

(c) introducing a transgene into the oocyte to produce a transgenic oocyte;

(d) *in vitro* fertilizing the transgenic oocyte to produce a zygote;

(e) transferring the zygote, or a cleaved embryo, morulae, or blastocyst formed from culturing said zygote into a recipient animal; and

(f) allowing the zygote, cleaved embryo, morulae, or blastocyst to develop to term.

In one embodiment of the above aspects of the invention, the transgene is introduced into the oocyte, zygote, or a cleaved embryo, morulae, or blastocyst formed from culturing the zygote by microinjection. In another embodiment, the transgene is introduced into the oocyte, zygote, or a cleaved embryo, morulae, or blastocyst formed from culturing the zygote by retroviral infection. In yet another embodiment, fertilization of the oocyte is done using transgenic semen.

In a fourth aspect, the invention features a method for generating a non-human transgenic animal, said method comprising:

(a) recovering an oocyte from a transgenic donor animal by

laparoscopic ovum pick up;

(b) *in vitro* maturing the oocyte;

(c) *in vitro* fertilizing the oocyte to produce a zygote;

(d) transferring the zygote, or a cleaved embryo, morulae, or blastocyst formed by culturing the zygote, into a recipient animal; and

(e) allowing the zygote, cleaved embryo, morulae, or blastocyst to develop to term.

In one embodiment of any of the above aspects of the invention, the animal is a ruminant, such as a goat or a sheep. In another embodiment, the donor and/or recipient animal is prepubertal.

By "transgenic animal" is meant a non-human animal containing a transgene.

By "transgene" is meant a DNA sequence that is introduced into the germline of a non-human animal by way of human intervention using any of the methods described herein, or methods known to one skilled in the art.

By "tissue-specific expression" is meant the expression within a specifically desired tissue, or a product released from a specific tissue. As used herein, "tissue-specific expression" refers to the production of a protein in the milk, urine, or blood specifically.

As referred to herein, by "prepubertal" is meant less than 5 months of age.

By "microinjection" is meant a procedure used for the introduction of material into a cell, involving injecting the material into the cell. As used herein, "microinjection" refers to DNA or retroviral particles introduced into an oocyte, zygote, cleaved embryo, morulae, or blastocyst.

By "retroviral infection" is meant a procedure used for the introduction of a retrovirus into a cell. As used herein, the retrovirus contains a transgene, and the result of retroviral infection is the introduction of the transgene into the cell.

The invention provides a number of advantages. The LOPU procedure can be repeated on the same animal for several months without a reduction in the number of oocytes recovered. Equally important is the fact that the LOPU procedure can be repeated in the same animal several times, because it does not leave surgical sequelae, as do standard procedures for recovering zygotes by laparotomy. In addition, the use of LOPU-derived oocytes results in a pregnancy rate which exceeds pregnancy rates achieved with *in vivo* produced and microinjected zygotes. This result was unexpected; literature would have led to the prediction that this system would be inefficient (Kühholzer and Brem, Theriogenology 51:1297-1302, 1999). Specifically, oocytes recovered from prepubertal donors have been reported to have a very low developmental capacity.

In addition, the ability to generate a transgenic animal using oocytes generated from a transgenic prepubertal donor is advantageous because it reduces the time required for propagating generations of transgenic animals.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawing

Figure 1 is a schematic representation of steps involved in the generation of transgenic animals using oocytes recovered by the LOPU procedure.

Detailed Description

Example 1

Synchronization and Gonadotrophin Stimulation of Goats to be Used as Donors of Oocytes Recovered by LOPU

Adult Goats: Adult goats may be subjected to LOPU without any hormonal stimulation. However, higher numbers of oocytes are obtained if donor goats are synchronized and stimulated with gonadotrophins. Synchronization of donor goats may be achieved using established protocols known to those skilled in the art. The following is an example of a synchronization protocol which may be used. Intravaginal sponges containing 60 mg of medroxyprogesterone acetate were inserted into the vagina of donor goats and left in place for 7 to 10 days, with an injection of 125 μg cloprostenol given 48 hours before sponge removal. Typically, for recovery of immature oocytes, the sponge was left in place until the time of oocyte collection, while for the recovery of oocytes which were more mature, the sponge was removed up to 48 hours before oocyte collection.

The priming of the ovaries was achieved using gonadotrophin preparations including follicle stimulating hormone (FSH), equine chorionic gonadotrophin (eCG), and human menopausal gonadotrophin (hMG). Any established regime for superovulation known by those skilled in the art may be used. The following hormonal regimes are examples of methods which may be used. A total dose equivalent to 120 mg of NIH-FSH-P1 was given twice daily in decreasing doses (35 mg/dose on the first day, 25 mg/dose on the second day) beginning 48 hours before sponge removal. Alternatively, 70 mg of NIH-FSH-P1 was given together with 400 IU of eCG 36 to 48 hours before LOPU. The recovered oocytes were then matured *in vitro* as described in Example 3.

An alternative strategy for the recovery of oocytes is to aspirate oocytes that have been matured *in vivo*. For this purpose it is essential to control the number of hours between the luteinizing hormone (LH) peak and the time at which the oocytes are collected. This may be achieved by drug-induced depletion of the endogenous LH peak. For example, the FSH/LH contents of the hypophysis may be depleted using gonadotrophin releasing hormone (GnRH) agonists such as buserelin or deslorelin. Alternatively, the hypophysis may be made refractory to hypothalamic GnRH using a GnRH antagonist such as cetrorelix. The desired GnRH agonist/antagonist may be administered by means of repeated injections, or more appropriately, by means of drug release devices such as subcutaneous implants or pumps. The GnRH agonist/antagonist is administered to the donor goats for at least 7 days prior to the start of gonadotrophin stimulation, and the treatment is continued until the LOPU procedure occurs. Follicular development is then stimulated by means of administration of gonadotrophins using a similar protocol as described above.

Prepubertal Goats: To recover oocytes from prepubertal goats, synchronization is not required. However, for recovering high numbers of oocytes, donor goats may need to be stimulated with gonadotrophin. This may be achieved by applying the same regimes used for superovulation of adult goats, as described above.

Example 2

Laparoscopic Ovum Pick-Up

Oocytes from donor goats were recovered by aspiration of follicle contents (puncture or folliculocentesis) under laparoscopic observation. The

laparoscopy equipment used (Richard Wolf, Germany) was composed of a 7 mm telescope, light cable, light source, 7 mm trocar for the laparoscope, atraumatic grasping forceps, and two 5 mm "second puncture" trocars. The follicle puncture set was composed of a puncture pipette, tubing, a collection tube, and a vacuum pump. The puncture pipette was made using a PVC pipette (5 mm external diameter, 2 mm internal diameter), and a 20G short bevel hypodermic needle, which was cut to a length of 5 mm and fixed into the tip of the pipette with instant glue. The connection tubing was made of silicon with an internal diameter of 5 mm, and connected the puncture pipette to the collection tube. The collection tube was a 50 ml centrifuge tube with an inlet and an outlet available in the cap. The inlet was connected to the pipette, and the outlet was connected to a vacuum line. Vacuum was provided by a vacuum pump connected to the collection tube by means of PVC 8 mm tubing. The vacuum pressure was regulated with a flow valve and measured as drops of collection media per minute entering the collection tube. The vacuum pressure was usually adjusted to 50 to 70 drops/minute.

The complete puncture set was washed and rinsed ten times with tissue culture quality distilled water before gas sterilization, and one time with collection medium before use. The collection medium was TCM 199 supplemented with 0.05 mg/ml of heparin and 1% (v/v) fetal calf serum (FCS). The collection tube contained approximately 0.5 ml of this medium to receive the oocytes.

The goats were fasted 24 hours prior to laparoscopy. Anaesthesia was induced by intravenous administration of diazepam (0.35 mg/kg body weight) and ketamine (5 mg/kg body weight), and maintained with isofluorane via endotrachial intubation. The animals were restrained in a cradle position for laparoscopic artificial insemination as described by Evans and Maxwell

(Salomon's Artificial Insemination of Sheep and Goats, Sydney: Butterworths, 1987). The 3 trocars described above were inserted and the abdominal cavity was filled with filtered air. The ovary was exposed by pulling the fimbria in different directions and the follicles were punctured. The needle was inserted into the follicle and rotated gently to ensure that as much of the follicle contents as possible were aspirated. After 3 to 5 follicles were aspirated, the pipette and tubing were rinsed using collection media from a sterile tube.

Results from LOPU procedures performed on two types of goats, standard dairy breeds (STD) and dwarf breeds (BELE), receiving hormonal treatments (as described in Example 1) in terms of number of follicles (FL) aspirated, and cumulus-oocyte complexes (COCs) recovered per donor (average ± standard deviation) are presented in Table 1.

Table 1. COCs Recovered from Goats Given Different Hormonal Treatments Prior to the LOPU Procedure

Breed	Sponge	Treatment	n	avg. FL	avg. COCs	% recovery
BELE	10 days	FSHx4	12	14.0±5.3	12.7±5.6	90.71
BELE	7-10 days	OS* 36h	15	11.2±5.0	10.0±5.7	89.29
BELE	7-10 days	OS 48h	14	11.7±5.4	10.1±5.6	86.32
STD	7-10 days	OS 36h	17	27.5±10.8	24.0±10.7	87.27
STD	7-10 days	OS 48h	14	24.4±8.9	20.1±6.9	82.38
STD	None	None	12	15.9±5.1	6.3±2.0	39.62

BELE©= Breed Early Lactate Early
STD= standard breed
OS* = one shot; all the gonadotrophic stimulation was given in a single injection, either 36 or 48 hours before LOPU

Example 3

Culture of Oocytes and *In Vitro* Production of Goat Zygotes Following LOPU Procedures

Oocyte Maturation: *In vitro* oocyte maturation (IVM) was done as follows. Cumulus-oocyte complexes (COCs) obtained by LOPU were washed in M199 supplemented with penicillin (100 IU/ml), streptomycin (0.1 mg/ml), kanamycin (0.05 mg/ml), and bovine serum albumin (BSA; 0.5%). COCs were placed into 1.5 ml vials containing 1 ml of the wash medium and transported to the laboratory in a thermos containing warm (34°C to 38°C) water. The COCs were then transferred into 50 μl drops of maturation medium, overlayed with mineral oil, and incubated at 38.5°C to 39°C for 24 to 27 hours in air containing 5% CO_2. An example of maturation medium consisted of M199 supplemented with bLH (0.02 U/ml; Sioux Biochemicals), bFSH (0.02 U/ml; Sioux Biochemicals), estradiol β-17 (1 μg/ml; Sigma), sodium pyruvate (0.2 mM; Sigma), kanamycin (50 μg/ml), and 10% heat-inactivated fetal calf serum (ImmunoCorp) or goat serum.

***In Vitro* Fertilization:** *In vitro* fertilization (IVF) was performed as follows. The expanded cumulus cells were partially or fully removed from the matured COCs by repeated pipetting with a fine bore pipette, dissection using 18 to 24G needles, or vortexing. Oocytes were washed in fertilization medium and transferred to drops of the same medium under mineral oil. Several protocols may be used for fertilization. For example, in one protocol the fertilization is SOFM (synthetic oviduct fluid medium) supplemented with penicillamine (3 μg/ml), hypotaurine (1 μg/ml), and 20% oestrus sheep serum. SOFM was the original synthetic oviduct fluid medium described by Tervit et al. (J. Reprod. Fertil. 30:493-497, 1972) and Gardner et al. (Biol. Reprod. 50:390-400, 1994),

further supplemented with glutamine (1 mM), BME-essential amino acids (EAA; 2% (v/v)) and MEM-nonessential amino acids (NEAA; 1% (v/v)). Five μl of the capacitated sperm suspension (10^6 sperm/ml) was added to the fertilization drop.

Sperm capacitation: Sperm capacitation may be accomplished using either fresh or frozen semen. The semen may be either transgenic or non-transgenic. Several protocols exist for this procedure. In one method, fresh semen was collected on the day of use. Motile sperm were separated on a 45%:90% Percoll gradient.

Alternatively, frozen semen was thawed in a sterile glass tube and was overlaid on top of a 45%:90% Percoll gradient. The resulting pellet was washed in HEPES-SOFM or mDM (glucose free -DM; Brackett, Biol. Reprod. 12:260-274, 1975) and resuspended in fertilization medium or mDM containing the appropriate capacitation factors. Capacitation may be performed using a variety of protocols. Examples of such protocols follow. The sperm were resuspended in either SOFM containing penicillamine (3 μg/ml), hypotaurine (1 μg/ml), and 20% oestrus sheep serum, or in mDM containing 8-bromo-cAMP (0.5 mM), ionomycin (100 nM), and heparin (10 μg/ml), or in mDM supplemented with calcium lactate (7.75 mM), caffeine (0.4 mg/ml), and heparin (10 μg/ml). The sperm concentration was then adjusted to a final concentration of 10^6 sperm/ml, and the sperm was added to fertilization drops containing the oocytes.

***In vitro* culture:** *In vitro* culture (IVC) of zygotes was achieved as follows. Seventeen to 19 hours after insemination, produced zygotes were placed into microdrops of 25 μl of low phosphate (0.35 mM) SOF embryo culture medium

under a mineral oil overlay. After 48 to 72 hours, cleaved embryos were moved to fresh microdrops of embryo culture medium. On day 4 or 5 (fertilization = day 0), embryos were moved to microdrops of high phosphate (1.2 mM) SOF embryo culture medium. Embryos were scored for blastocyst development on days 7 to 9.

Example 4

Transgenes Used for the Generation of Transgenic Goats and the Production of Heterologous or Homologous Protein in Milk, Urine, or Blood of the Transgenic Animal

A genetic construct suitable for use in the present invention generally includes the following elements:

(a) a promoter or transcription initiation regulatory unit,

(b) a transcription termination codon,

(c) DNA encoding a useful protein, or a nucleotide consisting of a ribozyme or antisense oligonucleotide,

(d) optionally, a naturally-occurring or provided sequence encoding a signal polypeptide directing the secretion of the recombinant protein from the cell if secretion is desired, or a naturally occurring or provided sequence, and

(e) optionally, an insulator element (e.g., chicken β-globin or chicken lysozyme MARS elements) that may result in a gene dosage effect (i.e., more copies of the transgene yield increased protein expression) or may allow for position-independent expression which is a result of the insulating effect from surrounding chromatin.

Conventional molecular biology methods may be used to generate and assemble the above elements and/or combinations thereof.

Milk-specific expression of a heterologous or homologous protein: Useful promoters include αs1-casein (as described, for example, in U.S. Patent No. 5,304,489), αs2-casein, β-casein, κ-casein, β-lactoglobulin (as described, for example, in U.S. Patent No. 5,322,773), α-lactalbumin, and whey acidic protein (WAP). If desired, the promoter may be linked to enhancer elements (such as CMV or SV40) or insulator elements (such as chicken β-globin).

An example of a DNA expression cassette using the WAP promoter, for example, as described in WO 92/22644, and insulator elements operably linked to a heterologous gene (in this case, a gene from a spider encoding components of spider silk) can be used as illustrated in WO 99/47661A2. This genetic construct also includes a transcription termination region. Preferably, but not limited, the termination region includes a poly-adenylation site at the 3' end of the gene from which the promoter region of the genetic construct was derived. The heterologous or homologous gene may be either a cDNA or genomic clone containing introns (all or a subset). If the gene is a cDNA clone, the genetic construct preferably also includes an intron which may increase the level of expression of the particular gene. Useful introns, for example, are those found in genes encoding caseins or immunoglobulins.

Urine-specific expression of a heterologous or homologous protein: Useful promoters for the urine-specific expression of a heterologous or homologous protein are those disclosed in PCT/US96/08233, and U.S. Patent No. 5,824,543, such as uroplakins I, II, and III, hereby incorporated by reference. The uroplakin II promoter, for example, has been shown to direct the expression of hGH in the urine of transgenic mice in detectable levels. Other useful promoters include kidney-specific promoters such as rennin and uromodulin.

Blood-specific expression of human immunoglobulin: A genetic construct that directs the blood-specific expression of human immunoglobulin may also be prepared. Such a construct would include human Ig loci containing plural variable V_h and V_k regions either as a mini-locus region or as a large portion of the Ig locus, as described in PCT/US97/23091 and references therein. Such a construct may be created using, for example, yeast artificial chromosomes (YACS) or mammalian artificial chromosomes.

In all of the above examples, the genetic construct may be microinjected into the pronucleus of a zygote or used to make retroviral particles which can infect an oocyte or zygote or embryo. The resulting embryo is transferred to a recipient (surrogate) mother and an offspring is born. This animal will have functional, mature B-cells expressing high affinity antibodies in the animal. Upon challenge with a specific antigen (e.g., anthrax) human antibodies will be produced, which may be purified from the plasma of these animals and subsequently used for the treatment of animals according to techniques described in WO 98/24893, hereby incorporated by reference.

Example 5

Microinjection of DNA into Zygotes Produced from LOPU-derived Oocytes

Zygotes produced as described in Example 3 are used in microinjection experiments in which one or both pronuclei are injected with a DNA construct of interest, as described in Example 4.

Pronuclear injection of DNA: Seventeen to 19 hours after insemination oocytes were observed for pronuclear (PN) formation using a microscope equipped with DIC optics. In order to improve PN visualization, oocytes may

be microcentrifuged. Zygotes with visible PN were microinjected with the DNA construct (1-2 ng/ml in a Tris-EDTA-NaCl buffer; pH 7.5) of interest. In general, transfer of embryos is preferably performed 2-4 hours following microinjection. For cases in which culture of embryos is preferred, the following protocol is an example of an *in vitro* culture method known to those skilled in the art.

***In vitro* culture:** Following microinjection, or after 17 to 19 hours post-insemination if microinjection is not desired, the zygotes were placed into microdrops of Gardner's G1 culture medium (Gardner and Lane, Human Reprod., Update 3:367-382, 1997) under an oil overlay. After 48 to 72 hours, cleaved embryos were moved to fresh microdrops of embryo culture medium. On day 4 or 5 (fertilization = day 0), embryos were moved to microdrops of Gardner's G2 medium. The embryos were scored for blastocyst development on days 7 to 9.

Embryo transfer: Injected embryos at the pronuclear or 2- to 8-cell stage may be transferred to the oviduct of synchronized recipients. If the embryos are at the morula or blastocyst stage they may be transferred to the uterus of synchronized recipients. Pregnancies were determined by ultrasound monitoring at 30 and 60 days of gestation.

Results:

Adult goats: Forty donors (of the Angora, Nubian, and Nigerian dwarf breeds) underwent LOPU following hormonal stimulation, and 459 COCs were recovered. Of those 459 COCs, 198 were developed to the PN stage, and 187 were microinjected with a transgene and subsequently transferred to 31

recipients, of which 23 became pregnant (74% initial pregnancy rate).

Prepubertal goats: Ten prepubertal Nubian goats were hormonally stimulated and oocytes were collected by LOPU (610 follicles were aspirated and 500 oocytes recovered). Twenty-four zygotes were transferred after IVF, to 4 recipients, of which, 2 became pregnant (50% initial pregnancy rate). Another set of 35 IVM/IVF-produced zygotes were microinjected and transferred to 5 recipients, of which, 3 became pregnant (60% initial pregnancy rate).

Example 6

Synchronization of Animals to be Used as Recipients of Embryos Derived From LOPU Procedures

Recipients are synchronized by any established regime known by those skilled in the art. They should be observed on standing heat during the day that the oocytes are *in vitro* fertilized. The following hormonal protocol is one example of a method which may be used. Intravaginal sponges containing 60 mg of medoxyprogesterone acetate were inserted into the vagina of recipient goats and left in place for 7 to 10 days with an injection of 125 μg closprostenol given 48 hours before sponge removal. Sponges were removed and an injection of 400 IU of eCG was administered on the same day as the LOPU took place.

Example 7

Transfer of Microinjected Embryos to Recipient Animals

Zygotes microinjected with the DNA expression cassette were either incubated for a short period (2-5 hours), overnight, or for 5 days, and then transferred to synchronized recipients. In each case, the microinjected embryos

may be cultured *in vitro* following any established protocol known by those skilled in the art. The following protocol is an example of one such method for the *in vitro* culture of microinjected embryos. Microinjected embryos were cultured under mineral oil, in 40 μl drops of culture medium consisting of SOFM supplemented with 8 mg/ml BSA-Fatty Acid Free. This *in vitro* culture was carried out at 39°C in a humidified incubator with 5% CO_2, 7% O_2, and 88% N_2. When longer *in vitro* culture was preferred, the embryos were moved every 48 hours into fresh drops of medium prepared 3 to 4 hours in advance.

The recipient goats were fasted 24 hours prior to surgery. Anesthesia was induced by intravenous administration of diazepam (0.35 mg/kg body weight) and ketamine (5 mg/kg body weight), and maintained with isofluorane via endotrachial intubation.

A laparoscopic exploration was then performed to confirm if the recipient had one or more recent ovulations/corpora lutea (CL) present in the ovaries and a normal oviduct and uterus. The laparoscopic exploration was carried out to avoid performing a laparotomy on an animal which had not responded properly to the hormonal synchronization protocol and to which an embryo should not be transferred. Following microinjection of the cassette, if a short (for example, 2-5 hours) or overnight culture period is preferred, the embryos may be transferred to the oviduct of recipient goats. For this purpose, a mid-ventral laparotomy of approximately 10 cm in length is established, the reproductive tract is exteriorized, and the embryos are implanted into the oviduct ipsilateral to ovulation/s by means of a TomCat catheter threaded into the oviduct from the fimbria.

If embryos are cultured for 5 days, the resulting morula/blastocyst-staged embryos may be transferred to the uterus. For this purpose, a mid-ventral laparotomy of approximately 5 cm in length is established and the

uterine horn ipsilateral to the CLs is exteriorized using a surgical clamp under laparoscopic observation. A small perforation is made with an 18G needle in the oviduct third of the horn, and the embryos are then implanted by means of a TomCat catheter threaded into the uterine lumen.

Example 8

Repeated LOPU Procedure Performed in Goats

Tables 2 and 3 summarize the results obtained when a series of LOPU procedures were performed on the same animals, as a demonstration of the reproducibility of the technique. The interval between collections was 30 to 60 days. These results demonstrate that the LOPU procedure can be repeated on the same animal for several months without a decrease in the number of oocytes recovered.

Table 2. Number of COCs Recovered per Goat (standard breed) Using Successive LOPU Procedures.

GOAT ID #	BREED	LOPU #1	LOPU #2	LOPU #3
24	ALP	16	22	
25	ALP	13	15	14
26	SAA	10	9	22
27	SAA	15	19	17
33	SAA	32	41	37
34	SAA	28	44	38
35	SAA	23	26	23
36	SAA	17	21	21
37	SAA	25	21	28
38	SAA	29	43	39
40	SAA	20	23	13
48	SAA	19	16	12
AVERAGE		20.58	25.00	24.00

ALP= Alpine, SAA= Saanen

Table 3. Number of COCs Recovered per Goat (Dwarf Breed) Using Successive LOPU Procedures

GOAT ID #	BREED	LOPU #1	LOPU #2
27	BELE	24	18
37	BELE	13	19
44	BELE	19	13
54	BELE	8	7
56	BELE	13	10
324	BELE	13	13
329	BELE	7	7
341	BELE	12	7
2	BELE	15	11
4	BELE	9	12
5	BELE	8	14
6	BELE	16	15
8	BELE	12	6
AVERAGE		13.00	11.69

Example 9

Infection of Oocytes Using Retroviruses

This example describes the introduction of exogenous DNA sequences into the genome of unfertilized oocytes recovered as described in the present invention. The DNA is introduced by microinjection of retroviruses into the peri-vitelline space of the oocytes.

The integration of retroviral genome into the genome of a pre-maturation or pre-fertilization oocyte followed by fertilization may be achieved as taught in WO 98/141,615, hereby incorporated by reference. The resulting embryo may be cultured or transferred to a recipient goat as described above.

Example 10

Pregnancies and Births Resulting from the Transfer of Embryos Derived from LOPU-recovered Oocytes

Morula and blastocyst-staged embryos derived from LOPU oocytes which underwent IVM/IVF/IVC were transferred into 2 recipients, one of which became pregnant giving birth to two normal kids (one male, one female).

Seven hundred twenty-one COCs were recovered from donor goats. After *in vitro* fertilization, 246 developed to the pronuclear stage, and of those, 222 were microinjected with a WAP-BIOSTEEL DNA construct and transferred to 36 synchronized recipients (an average of 6.2 embryos were transferred per recipient). Twenty recipients kidded 30 kids and of those 2 were transgenic for the WAP-BIOSTEEL construct, as tested by PCR and Southern blotting analyses. One of the transgenic kids was derived from an oocyte obtained from a pre-pubertal goat, while the other was derived from an oocyte obtained from an adult goat.

A summary of all of the recipient goat pregnancy rates now follows. With respect to oocytes derived from adult donor goats, 564 DNA microinjected zygotes were generated. These zygotes were transferred into 98 recipients (an average of 5.7 embryos per recipient), and 51 recipients became pregnant (54% pregnancy rate). With respect to oocytes derived from prepubertal donor goats, oocytes were recovered from 10 different donors. A total of 610 follicles were aspirated, and 500 oocytes were recovered. Twenty-

four non-microinjected zygotes were transferred to 4 recipients, of which, 2 became pregnant (50% pregnancy rate). One hundred forty-seven DNA microinjected zygotes were transferred into 25 recipients, and 11 recipients became pregnant (44% pregnancy rate).

These pregnancy rate results were unexpected as they exceeded pregnancy rates achieved with *in vivo* produced and microinjected zygotes. Although other reports (Kühholzer and Brem, supra) have found this *in vitro* fertilization system to be inefficient, these results indicate that this system is in fact efficient.

What is claimed is:

1. A method for generating a non-human transgenic animal, said method comprising:

(a) recovering an oocyte from a donor animal by laparoscopic ovum pick up;

(b) *in vitro* maturing said oocyte;

(c) *in vitro* fertilizing said oocyte to produce a zygote;

(d) introducing a transgene into said zygote, or a cleaved embryo, morulae, or blastocyst formed by culturing said zygote;

(e) transferring said zygote, cleaved embryo, morulae, or blastocyst into a recipient animal; and

(f) allowing said zygote, or morulae, or blastocyst to develop to term.

2. A method for generating a non-human transgenic animal, said method comprising:

(a) recovering an oocyte from a donor animal by laparoscopic ovum pick up;

(b) introducing a transgene into said oocyte to produce a transgenic oocyte;

(c) *in vitro* maturing said transgenic oocyte;

(d) *in vitro* fertilizing said transgenic oocyte to produce a zygote;

(e) transferring said zygote, or a cleaved embryo, morulae, or blastocyst formed from culturing said zygote into a recipient animal; and

(f) allowing said zygote, cleaved embryo, morulae, or blastocyst to develop to term.

3. A method for generating a non-human transgenic animal, said method comprising:

(a) recovering an oocyte from a donor animal by laparoscopic ovum pick up;

(b) *in vitro* maturing said oocyte;

(c) introducing a transgene into said oocyte to produce a transgenic oocyte;

(d) *in vitro* fertilizing said transgenic oocyte to produce a zygote;

(e) transferring said zygote, or a cleaved embryo, morulae, or blastocyst formed from culturing said zygote into a recipient animal; and

(f) allowing said zygote, cleaved embryo, morulae, or blastocyst to develop to term.

4. The method of claim 1, 2, or 3, wherein said transgene is introduced by microinjection.

5. The method of claim 1, 2, or 3, wherein said transgene is introduced by retroviral infection.

6. The method of claim 1, 2, or 3, wherein said fertilization is done using transgenic semen.

7. A method for generating a non-human transgenic animal, said method comprising:

(a) recovering an oocyte from a transgenic donor animal by laparoscopic ovum pick up;

(b) *in vitro* maturing said oocyte;

(c) *in vitro* fertilizing said oocyte to produce a zygote;

(d) transferring said zygote or a cleaved embryo, morulae or blastocyst formed by culturing said zygote, into a recipient animal; and

(e) allowing said zygote, cleaved embryo, morulae, or blastocyst to develop to term.

8. The method of claim 1, 2, 3, or 7, wherein said animal is a ruminant.

9. The method of claim 8, wherein said animal is a goat.

10. The method of claim 8, wherein said animal is a sheep.

11. The method of claim 1, 2, 3, or 7, wherein said animal is prepubertal.

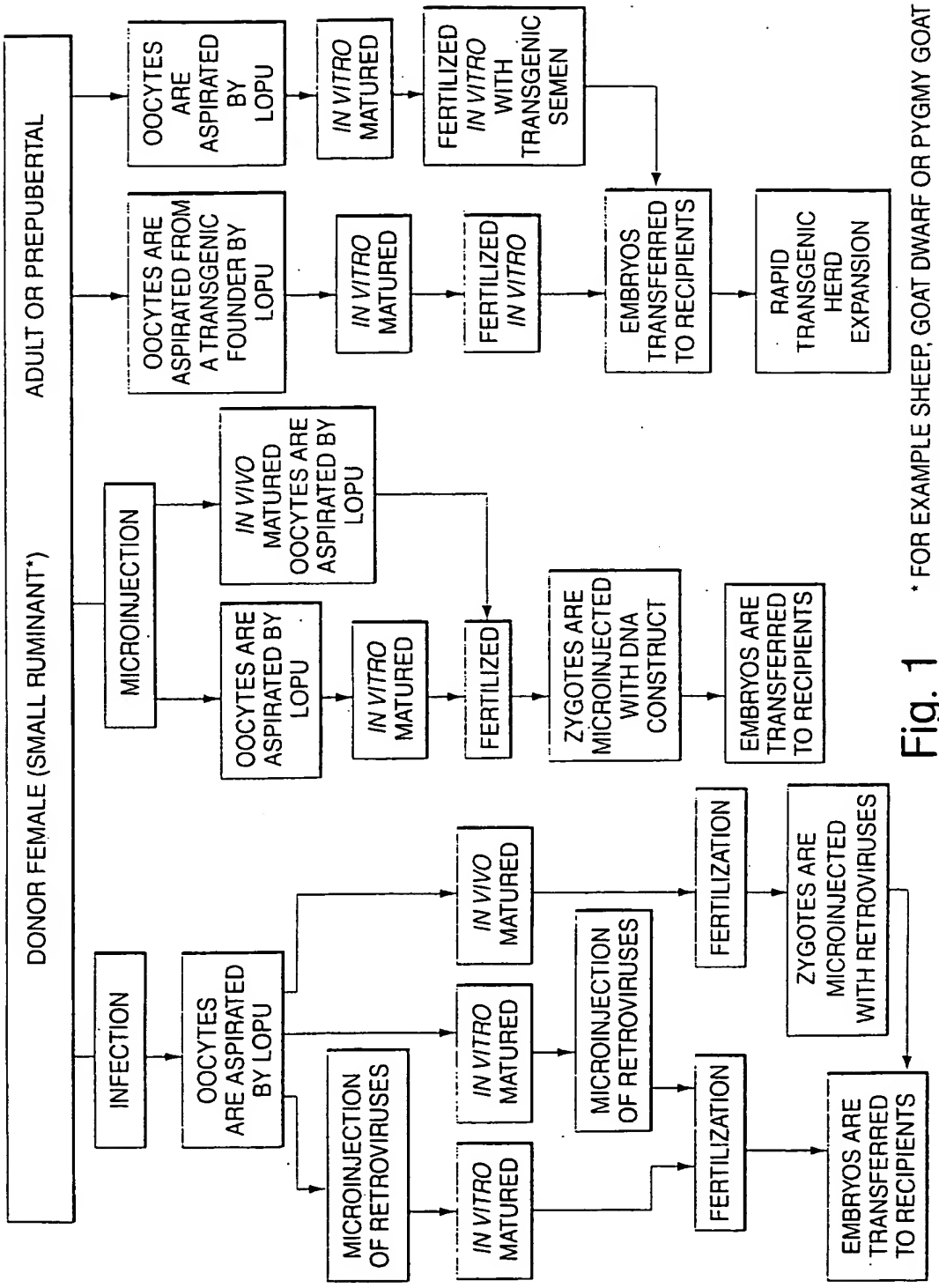

Fig. 1

* FOR EXAMPLE SHEEP, GOAT DWARF OR PYGMY GOAT
AGE: YOUNG OR ADULT

INTERNATIONAL SEARCH REPORT

Int. tional application No.
PCT/US00/29751

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :C12N 15/00, 15/09, 15/63, 5/00
US CL :800/21, 22, 23, 24, 25; 435/ 320.1, 325, 455
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/21, 22, 23, 24, 25; 435/ 320.1, 325, 455

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, MEDLINE, NPL
search terms: laparoscopic, ovum, ova, oocyte, transgenic, zygote, embryo, morulae, blastocyst

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,693,534 A (ALAK et al.) 02 December 1997, cols. 11-12	1-11
Y, P	US 6,110,741 A (HEARN) 29 August 2000, col. 3, lines 53-58.	1-11
Y	US 5,907,080 A (KARATZAS et al.) 25 May 1999, see entire document.	1-11
Y	US 5,366,888 A (FRY et al.) 22 November 1994, see col. 3.	1-11
Y	US 5,895,833 A (BERG) 20 April 1999, see entire document.	1-11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 FEBRUARY 2001	30 MAR 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JILL D. MARTIN Telephone No. (703) 308-0196

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